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(54) Title: APO–2 LIGAND–ANTI–HER–2 ANTIBODY SYNERGISM

(57) Abstract

Methods of using synergistically effective amounts of Apo–2 ligand and anti–Her–2 antibodies to enhance cell death via apoptosis are provided.

Apo-2 LIGAND-ANTI-Her-2 ANTIBODY SYNERGISM

Field of the Invention

This invention relates generally to methods of inducing apoptosis in mammalian cells. In particular, it pertains to the use of Apo-2 ligand and anti-Her-2 antibody to synergistically induce apoptosis in mammalian cells.

Background of the Invention

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, *e.g.*, Barr et al., Bio/Technology, 12:487-493 (1994)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death, however, have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, Science, 267:1445-1449 (1995); Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

Various molecules, such as tumor necrosis factor- α ("TNF-α "), tumor necrosis factor-β ("TNF-β " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, and Apo-1 ligand (also referred to as Fas ligand or CD95 ligand) have been identified as members of the tumor necrosis factor ("TNF")

Baselga et al., Pharmac. Ther., 64:127-154 (1994); Masui et al., Cancer research, 44:1002-1007 (1984).

The second member of the class I subfamily, p185*neu*, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the *neu* protooncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of *neu* (called Her-2 or *erb*B2) is observed in breast and ovarian cancers and generally correlates with a poor prognosis [Slamon et al., Science, 235:177-182 (1987); Slamon et al., Science, 244:707-712 (1989)]. Accordingly, Slamon et al. in U.S. Pat. No. 4,968,603 describe various diagnostic assays for determining Her-2 gene amplification or expression in tumor cells. To date, no point mutation analogous to that in the *neu* protooncogene has been reported for human tumors. Overexpression (frequently but not uniformly due to amplification) of Her-2 has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King et al., Science, 229:974 (1985); Yokota et al., Lancet, 1:765-767 (1986); Fukushigi et al., Mol. Cell. Biol., 6:955-958 (1986); Geurin et al., Oncogene Research, 3:21-31 (1988); Cohen et al., Oncogene, 4:81-88 (1989); Yonemura et al., Cancer Research, 51:1034 (1991); Borst et al., Gynecol. Oncol., 38:364 (1990); Weiner et al., Cancer Research, 50:421-425 (1990); Kern et al., Cancer Research, 50:5184 (1990); Park et al., Cancer Research, 49:6605 (1989); Zhau et al., Mol. Carcinog., 3:354-357 (1990); Aasland et al., Br. J. Cancer, 57:358-363 (1988); Williams et al., Pathobiology, 59:46-52 (1991); and McCann et al., Cancer, 65:88-92 (1990).

Certain antibodies directed against the rat *neu* and human Her-2 protein products have been described. Drebin et al., Cell, 41:695-706 (1985) refer to an IgG2a monoclonal antibody which is directed against the rat *neu* gene product. This antibody called 7.16.4 causes down-modulation of cell surface p185 expression on B104-1-1 cells (NIH-3T3 cells transfected with the *neu* protooncogene) and inhibits colony formation of these cells. In Drebin et al., Proc. Natl. Acad. Sci., 83:9129-9133 (1986), the 7.16.4 antibody was shown to inhibit the tumorigenic growth of *neu*-transformed NIH-3T3 cells as well as rat neuroblastoma cells (from which the *neu* oncogene was initially isolated) implanted into nude mice. Drebin et al., Oncogene, 2:387-394 (1988) discuss the production of a panel of antibodies against the rat *neu* gene product. All of the antibodies were found to exert a cytostatic effect on the growth of *neu*-transformed cells suspended in soft agar. Antibodies of the IgM, IgG2a and IgG2b isotypes were able to mediate *in vitro* lysis of *neu*-

antibodies are not thought to induce apoptosis by disruption of an autocrine loop.

Other antibodies specific for Her-2 have been described in the art. Tagliabue et al., Int. J. Cancer, 47:933-937 (1991) describe two antibodies which were selected for their reactivity on the lung adenocarcinoma cell line (Calu-3) which overexpresses Her-2. One of the antibodies, called MGR3, was found to internalize, induce phosphorylation of Her-2, and inhibit tumor cell growth *in vitro*.

McKenzie et al., Oncogene, 4:543-548 (1989) generated a panel of anti-Her-2 antibodies, including the antibody designated TA1. This TA1 antibody was found to induce accelerated endocytosis of Her-2 [see Maier et al., Cancer Research, 51:5361-5369 (1991)]. Bacus et al., Mol. Carcinogenesis, 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the Her-2 gene) and MCF-7. Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of Her-2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski et al., Proc. Natl. Acad. Sci., 88:8691-8695 (1991) generated a panel of anti-Her-2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the Her-2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the Her-2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-Her-2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis *in vitro* via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus et al., Cancer Research, 52:2580-2589 (1992) further characterized the antibodies described in Bacus et al. (1990) and Stancovski et al. cited above. Extending the i.p. studies of Stancovski et al., the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human Her-2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the Her-2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus et al. also

SUMMARY OF THE INVENTION

Applicants have surprisingly found that Apo-2 ligand and anti-Her-2 antibody can act synergistically to induce apoptosis in mammalian cells, particularly in mammalian cancer cells which overexpress Her-2.

The invention provides various methods for the use of Apo-2 ligand and anti-Her-2 antibody to induce apoptosis in mammalian cells. For example, the invention provides a method for inducing apoptosis comprising exposing a mammalian cell, such as a cancer cell which overexpresses Her-2, to Apo-2 ligand and anti-Her-2 antibody in an amount effective to synergistically induce apoptosis. The cell may be in cell culture or in a mammal, *e.g.* a mammal suffering from cancer. Thus, the invention includes a method for treating a mammal suffering from a condition characterized by overexpression of the Her-2 receptor, comprising administering an effective amount of Apo-2 ligand and anti-Her-2 antibody, as disclosed herein. According to any of the methods, one or more anti-Her-2 antibodies may be used. For instance, a first anti-Her-2 antibody such as the 7C2 antibody and a second anti-Her-2 antibody (different from the first antibody such as an antibody which binds to a different Her-2 epitope) may be employed. Preferably, at least one of the anti-Her-2 antibodies is an apoptosis-inducing antibody. Optionally, the methods may employ an agonistic anti-Apo-2 ligand receptor antibody which mimics the apoptotic activity of Apo-2 ligand.

The invention also provides compositions which comprise Apo-2 ligand and/or anti-Her-2 antibody(s). Optionally, the compositions of the invention will include pharmaceutically acceptable carriers or diluents. Preferably, the compositions will include Apo-2 ligand and/or anti-Her-2 antibody in an amount which is effective to synergistically induce apoptosis in mammalian cells.

The invention also provides articles of manufacture and kits which include Apo-2 ligand and/or anti-Her-2 antibody(s).

Brief Description of the Drawings

Figure 1A shows a bar diagram illustrating the enhanced apoptotic activity (as determined by annexin V binding and uptake of PI) of Apo-2L and 7C2 antibody on BT474 and MCF7/HER2 breast tumor cells.

Figure 1B shows a bar diagram illustrating the enhanced apoptotic activity (as determined by annexin V binding and uptake of PI) of Apo-2L and 7C2 antibody on SKBR3 breast tumor cells.

Figure 2A shows a bar graph showing the decrease in SKBR3 viable cell number and the increased number of dead cells (as measured by trypan blue dye

recombinant or synthetic methods. The term Apo-2 ligand also refers to the polypeptides described in WO 97/25428, supra.

Unless indicated otherwise, the term "Her-2" when used herein refers to human Her-2 protein and human Her-2 gene. The human Her-2 gene and Her-2 protein are described in Semba et al., Proc. Natl. Acad. Sci., 82:6497-6501 (1985) and Yamamoto et al., Nature, 319:230-234 (1986) (Genebank accession number X03363), for example. Her-2 comprises four domains (Domains 1-4). "Domain 1" is at the amino terminus of the extracellular domain of Her-2. See Plowman et al., Proc. Natl. Acad. Sci., 90:1746-1750 (1993).

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of Her-2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on Her-2 (*i.e.* any one or more of residues in the region from about residue 22 to about residue 53 of Her-2).

The "epitope 4D5" is the region in the extracellular domain of Her-2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of Her-2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of Her-2 (*i.e.* any one or more residues in the region from about residue 529, *e.g.* about residue 561 to about residue 625, inclusive).

A cell which "overexpresses" Her-2 has significantly higher than normal Her-2 levels compared to a noncancerous cell of the same tissue type. Typically, the cell is a cancer cell, *e.g.* a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. The cell may also be a cell line such as SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3.

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the Her-2-ErbB3 and Her-2-ErbB4 protein complexes (*i.e.* induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., Science, 256:1205-1210 (1992); WO 92/20798; Wen et al., Mol. Cell. Biol., 14(3):1909-1919

three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., NIH Publ. No.91-3242, Vol. I, pages 647-669 (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of

sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "ScFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the ScFv to form the desired structure for antigen binding. For a review of ScFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H -

II. Methods and Materials

Generally, the methods of the invention for inducing apoptosis in mammalian cells comprise exposing the cells to an effective amount of Apo-2 ligand and anti-Her-2 antibody. Preferably, the amount of Apo-2L and anti-Her-2 antibody employed will be amounts effective to synergistically induce apoptosis. This can be accomplished *in vivo* or *ex vivo* in accordance, for instance, with the methods described below and in the Example. It is contemplated that the present invention may be used to treat various conditions, including those characterized by overexpression and/or activation of the Her-2 receptor. Exemplary conditions or disorders to be treated with the Apo-2 ligand and anti-Her-2 antibody include benign or malignant cancer. Methods of determining levels of Her-2 expression prior to exposing cells to Apo-2 ligand and anti-Her-2 antibody are well known in the art. For example Slamon et al. in U.S. Pat. No. 4,968,603 describe various diagnostic assays for determining *Her-2* gene amplification or expression in tumor cells.

A. MATERIALS

The Apo-2L which can be employed in the methods includes the Apo-2L polypeptides described in Pitti et al., supra, and WO 97/25428, supra. It is contemplated that various forms of Apo-2L may be used, such as the full length polypeptide as well as soluble forms of Apo-2L which comprise an extracellular domain (ECD) sequence. Examples of such soluble ECD sequences include polypeptides comprising amino acids 114-281, 91-281 or 92-281 of the Apo-2L sequence shown in Figure 1A of Pitti et al., J. Biol. Chem., 271:12687-12690 (1996). It is presently believed that the polypeptide comprising amino acids 92-281 is a naturally cleaved form of Apo-2L. Applicants have expressed human Apo-2L in CHO cells and found that the 92-281 polypeptide is the expressed form of Apo-2L. Modified forms of Apo-2L, such as the covalently modified forms described in WO 97/25428 are included. In particular, Apo-2L linked to a non-proteinaceous polymer such as polyethylene glycol is included for use in the present methods. The Apo-2L polypeptide can be made according to any of the methods described in WO 97/25428.

It is contemplated that a molecule which mimics the apoptotic activity of Apo-2L may alternatively be employed in the presently disclosed methods. Examples of such molecules include agonistic antibodies which can induce apoptosis in a like manner to Apo-2L. In particular, these agonist antibodies would comprise antibodies to one or more of the receptors for Apo-2L and which can stimulate apoptosis. Agonist antibodies directed to at least one of these receptors,

epitope on Her-2 to which 4D5 antibody binds.

The Her-2 antigen to be used for production of antibodies may be, *e.g.*, a soluble form of the extracellular domain of Her-2; a peptide such as a Domain 1 peptide or a portion thereof (*e.g.* comprising the 7C2 or 7F3 epitope). Alternatively, cells expressing Her-2 at their cell surface; or a carcinoma cell line such as SKBR3 cells, see Stancovski et al., PNAS (USA), 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of Her-2 useful for generating antibodies will be apparent to those skilled in the art.

To identify or select for antibodies which induce apoptosis, loss of membrane integrity as indicated by, *e.g.*, PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection (Manassas, VA)) are cultured in Dulbecco's Modified Eagle Medium (D-MEM):Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 10^6 per dish in 60 x 15 mm dishes and allowed to attach 2-3 days. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4^0C, the pellet resuspended in 1 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1 ml per tube) for removal of cell clumps. Tubes then receive PI (0.1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of apoptosis as determined by PI uptake can be selected.

In order to select for antibodies which induce apoptosis, one can perform an annexin binding assay using BT474 cells as described in the Example below. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labelled annexin (*e.g.* annexin V-FITC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer

serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase

into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., Curr. Opinion in Immunol., 5:256-262 (1993) and Pluckthun, Immunol. Revs., 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990). Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., Bio/Technology, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse et al., Nuc. Acids. Res., 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, et al., Proc. Natl Acad. Sci. USA, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized and human antibodies

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a

it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581-597 (1991)).

(iv) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto et al., Journal of Biochemical and Biophysical Methods, 24:107-117 (1992) and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology, 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the Her-2 protein. For example, one arm may bind an epitope in Domain 1 of Her-2 such as the 7C2/7F3 epitope, the other may bind a different Her-2 epitope, *e.g.* the 4D5 epitope. Other such antibodies may combine a Her-2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-Her-2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the Her-2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express Her-2. These antibodies possess an Her-2-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain,

unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986). According to another approach described in W096/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby et

thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al., Anti-Cancer Drug Design, 3:219-230 (1989).

(vii) Antibody-salvage receptor binding epitope fusions.

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.* by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased *in vivo* half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer *in vivo* half-life than that of the original antibody. If the antibody variant does not have a longer *in vivo* half-life upon testing, its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer *in vivo* half-life, and this process is continued until a molecule is obtained that exhibits a longer *in vivo* half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, *e.g.*, on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

B. FORMULATIONS

The Apo-2 ligand and anti-Her-2 antibody are preferably administered in

particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

C. MODES OF ADMINISTRATION

The Apo-2L and Her-2 antibody can be administered in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Optionally, administration may be performed through mini-pump infusion using various commercially available devices.

Effective dosages and schedules for administering Apo-2 ligand and Her-2 antibody may be determined empirically, and making such determinations is within the skill in the art. It is presently believed that an effective dosage or amount of Apo-2 ligand used alone may range from about 1 μg/kg to about 100 *mg*/kg of body weight or more per day. Interspecies scaling of dosages can be performed in a manner known in the art, e.g., as disclosed in Mordenti et al., Pharmaceut. Res., 8:1351 (1991). Those skilled in the art will understand that the dosage of Apo-2 ligand that must be administered will vary depending on, for example, the mammal which will receive the Apo-2 ligand, the route of administration, and other drugs or therapies being administered to the mammal.

Depending on the type of cells and/or severity of the disease, about 1 μg/kg to 15 mg/kg (*e.g.* 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100

employed for the Apo-2 ligand or Her-2 antibody, or it may be administered via a different mode.

Radiation therapy can be administered according to protocols commonly employed in the art and known to the skilled artisan. Such therapy may include cesium, iridium, iodine, or cobalt radiation. The radiation therapy may be whole body irradiation, or may be directed locally to a specific site or tissue in or on the body. Typically, radiation therapy is administered in pulses over a period of time from about 1 to about 2 weeks. The radiation therapy may, however, be administered over longer periods of time. Optionally, the radiation therapy may be administered as a single dose or as multiple, sequential doses.

The Apo-2 ligand and anti-Her-2 antibody (and one or more other therapies) may be administered concurrently or sequentially. Following administration of Apo-2 ligand and Her-2 antibody, treated cells *in vitro* can be analyzed. Where there has been *in vivo* treatment, a treated mammal can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically, by biopsy or by standard x-ray imaging techniques.

III. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agents in the composition are the Apo-2 ligand and anti-Her-2 antibodies. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

were detached by trypsinization and pooled with the corresponding supernatant. Cells were then centrifuged at 1200 rpm for 5 minutes at 4°C, the pellet resuspended in 1 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1 ml per tube) for removal of cell aggregates. Each tube then received annexin V-FITC (0.1 µg/ml) or PI (10 µg/ml) or annexin V-FITC plus PI or trypan blue. Samples were analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson).

The results of the experiments are shown in Figures 1-3. Combinations of the Apo-2 ligand and the 7C2 anti-Her-2 MAb synergistically induced apoptosis in the cell lines which overexpress Her-2 as evidenced by annexin-V binding, PI uptake or trypan blue uptake.

Deposit of Materials

The following materials have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	October 17, 1996
7F3	ATCC HB-12216	October 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
Apo-2L	ATCC CRL 12014	January 3, 1996

These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of deposit. The deposits will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures (a) that access to the cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR §1.14 and 35 USC §122, and (b) that all restrictions on the availability to the public of the cultures so deposited will be irrevocably removed upon the granting of the patent.

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposits is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Applicant's or agent's file reference 11669.20WO01	International application No. PCT/US 99 / 06673

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 33, line 18

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*

11801 University Boulevard
Manassas, Virginia USA

Date of deposit	Accession Number
October 17, 1996	ATCC HB-12215

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Authorized officer: Hal Saunders	Authorized officer

Form PCT/RO/134 (July1998)

Applicant's or agent's file reference 11669.20W001	International application No. PCT/US 99 / 06673

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 33, line 20

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
11801 University Boulevard
Manassas, Virginia USA

Date of deposit	Accession Number
May 24, 1990	ATCC CRL 10463

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Hal Saunders	Authorized officer

Form PCT/RO/134 (July 1998)

WHAT IS CLAIMED IS:

1. A method of inducing apoptosis in cancer cells comprising exposing mammalian cancer cells expressing Her-2 receptor to a synergistically effective amount of Apo-2 ligand and anti-Her-2 antibody.

2. The method of claim 1 wherein the Apo-2 ligand is linked to a nonproteinaceous polymer.

3. The method of claim 2 wherein the polymer is polyethylene glycol.

4. The method of claim 1 wherein the anti-Her-2 antibody is an apoptotic antibody.

5. The method of claim 1 wherein the anti-Her-2 antibody binds to Domain 1 of Her-2.

6. The method of claim 1 wherein the anti-Her-2 antibody binds to epitope 7C2 on Her-2.

7. The method of claim 1 wherein the anti-Her-2 antibody is a monoclonal antibody.

8. The method of claim 1 wherein the anti-Her-2 antibody has nonhuman complementarity determining region (CDR) residues and human framework region (FR) residues.

9. The method of claim 1 wherein the anti-Her-2 antibody has complementarity determining regions (CDRs) of antibody 7C2.

10. The method of claim 1 wherein the anti-Her-2 antibody is a bispecific antibody.

11. The method of claim 10 wherein the bispecific antibody comprises an antibody having one arm which binds a Her-2 epitope and another arm which binds a receptor for Apo-2 ligand.

an Apo-2 ligand contained within the container; and
an anti-Her-2 antibody contained within the container;
wherein the Apo-2 ligand and anti-Her-2 antibody are present in synergistically effective amounts and the label on the container indicates that combinations of the Apo-2 ligand and the anti-Her-2 antibody can be used for treating cancer cells which overexpress Her-2.

FIG. 2A

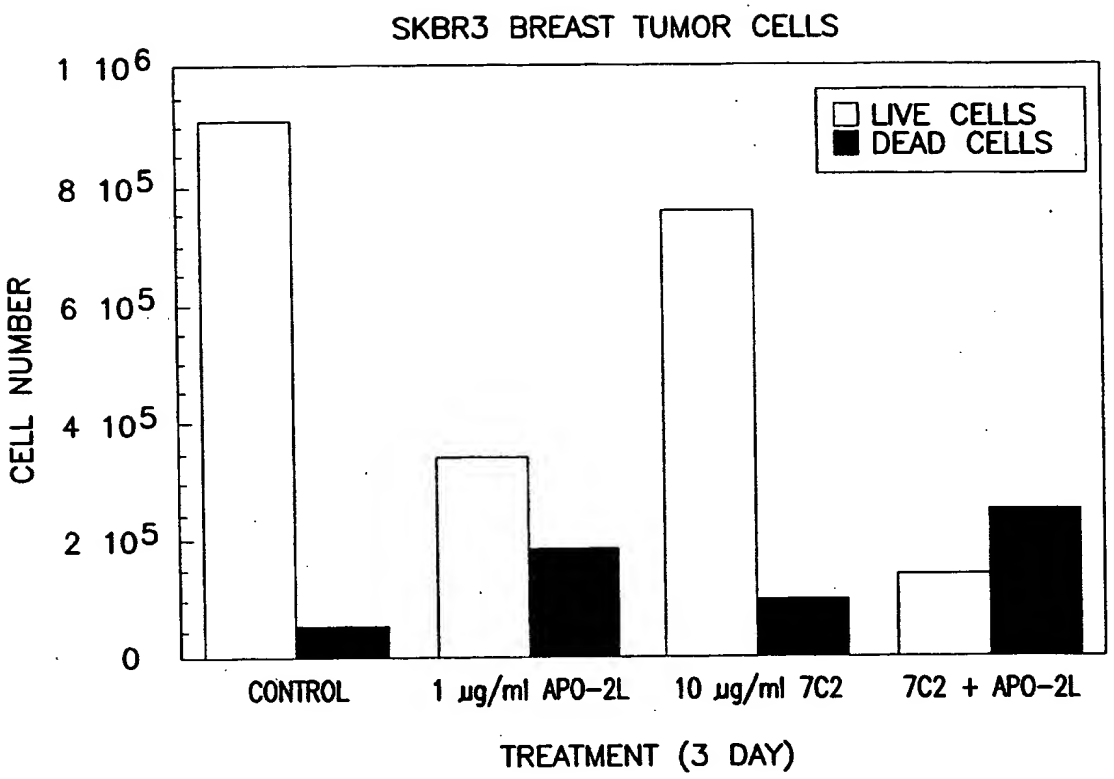
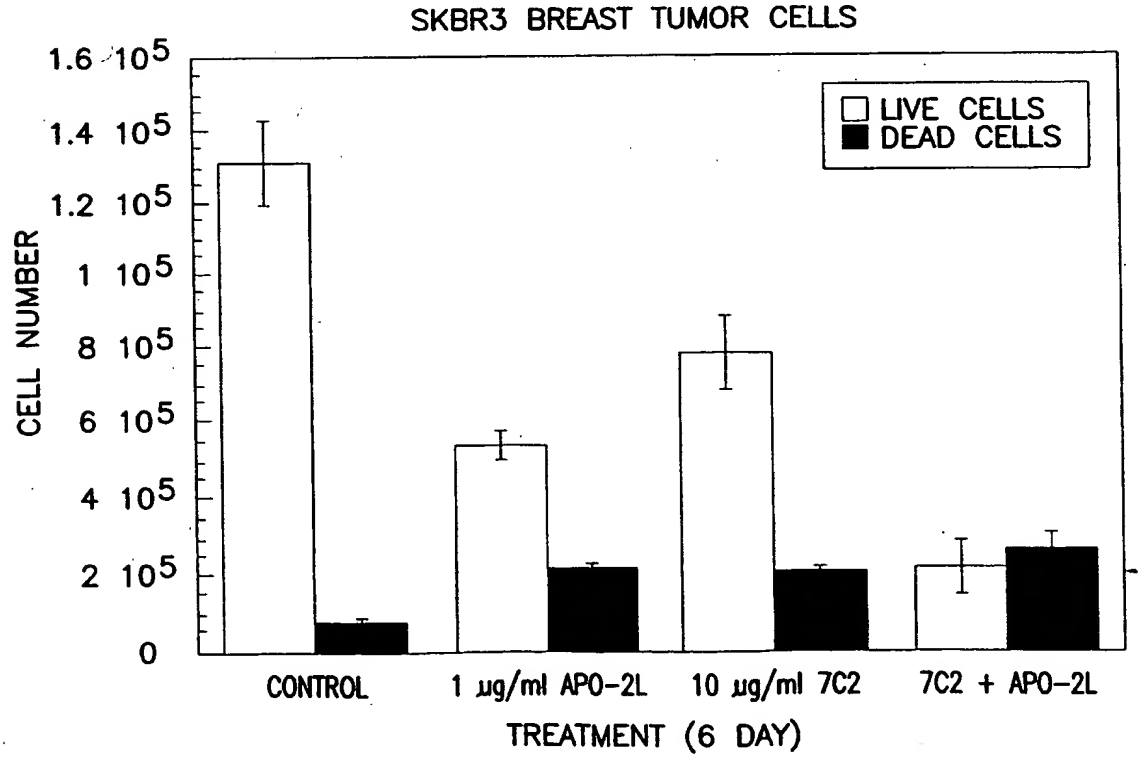


FIG. 2B



INTERNATIONAL SEARCH REPORT

Interr ial Application No
PCT/US 99/06673

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/17 //C07K14/705,C07K16/32,(A61K39/395, 38:17)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHEPARD M H ET AL: "Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic." JOURNAL OF CLINICAL IMMUNOLOGY, vol. 11, no. 3, 1991, pages 117-127, XP000560916 see abstract; figure 1 ---	1-23
A	EP 0 656 367 A (BECTON DICKINSON AND CIE) 7 June 1995 see claims 1-7; table 1 ---	1-23

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1999	02/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Flao, K

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir. ational application No.
PCT/US 99/06673

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-16
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.